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Structural basis for phospholipid scrambling in the TMEM16 family

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Upon activation, lipid scramblases dissipate the lipid asymmetry of membranes, in an ATP-independent manner, by catalyzing flip-flop of lipids between the leaflets. The molecular identities of these proteins long remained obscure, but in recent years the TMEM16 family of proteins has been found to constitute Ca^{2+}-activated scramblases. Recently, the X-ray structure of a fungal TMEM16 homologue has provided insight into the architecture of this protein family and into potential scrambling mechanisms. The protein forms homodimers with each subunit containing a membrane-spanning hydrophilic cleft. This region is of sufficient size to harbor

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Introduction

In a typical animal cell plasma membrane (PM) the different lipid species that make up the bulk of the membrane are not evenly distributed between both leaflets. The extracellular leaflet contains an excess of phosphatidylcholine (PC) and sphingomyelin (SM) relative to the inner leaflet, whereas the cytosolic leaflet is enriched in phosphatidyletholamine (PE) and phosphatidylserine (PS) [1,2]. In intracellular membrane compartments the degree of this asymmetry can deviate substantially from the PM (Figure 1a and b). For example, in the endoplasmic reticulum (ER) the lipid distribution is thought to be nearly symmetric [3] (but see also reference [4]). Spontaneous movement of lipids between the two leaflets, termed flip-flop, is naturally hindered by the large energy barrier for the hydrated lipid headgroup upon traversing the hydrophobic core of the bilayer and occurs at a time scale of hours [5]. The distribution of different membrane lipids is partly a result of synthesis (as is the case of SM [6]) but mainly the consequence of transport processes by means of specialized ATP-driven membrane transporters [7]. Members of the P4-type ATPase family (flippases) are responsible for transporting PE and PS to the cytosolic face, while the activity of certain ABC transporters (floppases) results in net export of PC to the outer leaflet [8-15] (Figure 1c). Lipid scramblases, the subject of this review, are an exception in that rather than maintaining bilayer asymmetry they randomize lipid distribution across both leaflets. Scramblase function is ATP-independent, facilitating passive, non-specific lipid transport that effectively disperses the bilayer in response to specific cellular cues [16] (Figure 1c). As a consequence, inner leaflet lipids such as PS move to the outer leaflet, serving as a trigger for apoptosis [17,18] or blood clotting [19]. Furthermore, in the ER scrambling processes are believed to mediate the redistribution of newly synthesized lipid molecules across the leaflets and are also required to provide components for protein N-glycosylation at the luminal side [20]. The coordinated transport of lipids through flippases, floppases and scramblases has thus impact on basic features of a cell such as signaling, trafficking and the determination of membrane curvature, besides numerous other functionalities [21-25].

While the triggered breakdown of lipid asymmetry at the plasma membrane has been known for decades [26], the underlying molecular identities of involved proteins are only beginning to emerge. It is has by now become clear that the scramblase function is not restricted to a single class of proteins [16,20,27•,28••]. One family that includes Ca^{2+} activated lipid scramblases is the TMEM16 family of proteins [29••] (Figure 1d). Recently, the first structure of a TMEM16 lipid scramblase (nhTMEM16) has been elucidated [30••] (Figure 2). This structure confirmed

predicted features of scramblases [31,32] and it provided detailed insights on the scrambling mechanism itself. In this review we discuss the nhTMEM16 structure with respect to the function of scramblases in general as well as for the TMEM16 family in particular. Aspects regarding the channel function found in certain TMEM16 proteins have been reviewed recently [33-36] and only overlapping functionalities will be addressed.

Ca^{2+}-activated lipid scrambling by a TMEM16 family member

The TMEM16 family of proteins, also known as anoctamins, has been identified less than a decade ago to contain the long sought-after Ca^{2+}-activated Cl^- channels (CaCCs) [37-39]. Based on this discovery, it was initially assumed that all homologues might share a similar function. It was therefore a big surprise when TMEM16F, the factor underlying the exposure of PS upon Ca^{2+} activation in blood platelets, was found to be a member of the same family [28••]. Non-functional TMEM16F has been shown to be one possible cause of Scott-Syndrome [28••], a rare bleeding disorder caused by the disruption of the blood–clotting cascade [40]. The link between TMEM16F and PS exposure pointed to a role for TMEM16 proteins in bilayer scrambling. The question of whether this is achieved indirectly through ion channel activity, or directly, with the implication that TMEM16F is itself a scramblase, has proved contentious [34,41•]. This dispute could be resolved with fungal homologues that, after purification and reconstitution into liposomes, allowed confirmation of their scrambling activity *in vitro* [30••,42••]. In conjunction with investigations on other TMEM16 members in cellular systems [29••], these experiments convincingly supported the existence of a scrambling function in the TMEM16 family. There is still an ongoing debate as to whether scramblases can function as channels, and vice versa, but consensus has now been reached on the duality within this particular family of proteins [34,43]. A similar duality is also found in case of P-type ATPases where related family members constitute primary active ion pumps or lipid transport proteins.

Overall structure of nhTMEM16

The structure of nhTMEM16, displayed in Figure 2, has revealed the common architecture of this protein family. TMEM16 proteins form dimers with ten transmembrane helices in each subunit. They show no similarity to any other membrane protein structure and also do not contain any internal symmetry of helices frequently found in transport proteins (Figure 2c).

While the dimeric organization has been recognized before [44,45], the annotation of eight transmembrane segments (hence the name anoctamins) proved to be wrong [39]. This mismatch is likely due to the unusually high hydrophilicity of some transmembrane segments [30••]. Within the membrane, the dimer interface is comparably small and the arrangement of helices creates a spacious cavity between both subunits (termed dimer cavity) that is probably filled with lipids (Figure 2c). Both subunits interact via a glutamate and a histidine at the luminal side, residues which are conserved in most family members (Figure 2d) except for TMEM16A/B, where the presumably charged residues are replaced by an uncharged glutamine and asparagine. This difference may disfavor heterodimerization of the ion channels with the putative scramblases TMEM16C-K. Consequently, TMEM16A and B can form mutual heterodimers but fail to do so with TMEM16F [46]. Previous experiments have proposed direct interactions between both N-termini in the dimer [46], which is not consistent with the structure. Instead, in nhTMEM16, the C-terminus of one subunit wraps around the N-terminus of the other, thus constituting a large portion of the interaction interface (Figure 2e). While both termini constitute the cytosolic part of the protein, no clear role in protein function could so far be assigned to this region. The most intriguing architectural features in nhTMEM16 are a membrane-exposed hydrophilic cavity and a calcium binding site located within the bilayer, both of which are described in detail below.

A hydrophilic groove suited to accommodate lipid headgroups

A hydrophilic and membrane-exposed surface that spans the bilayer from the cytosolic to the luminal side has previously been predicted as a requirement for scramblases [31,32]. Strikingly, such a feature is found in the crystal structure of nhTMEM16 [30••]. In this protein, two spirally wound hydrophilic grooves (the subunit cavities) that are each contained within a single subunit and located at the opposite ends of the dimer are laterally exposed to the membrane (Figure 2b, c and f). Although lipids were not visible in the structure, these grooves appear ideally suited to harbor lipid headgroups. On the hydrophilic surface, the headgroups could traverse the width of the entire membrane, like on a slide, without being exposed to the hydrophobic core of the bilayer, while the alkyl moieties of the lipids would remain in their favorable hydrophobic environment. During this process the energy barrier for the flip-flop of a lipid would be strongly reduced and rates therefore greatly enhanced.

Most research on TMEM16 proteins has so far been conducted on the Cl^- channel TMEM16A prior to the availability of structural data. Numerous experiments, while aimed at revealing the ion conduction path, also provide indications where the scrambling of lipids takes place. Extensive mutational studies have identified residues around the luminal entrance of the subunit cavity to influence ion conduction [47•] and a corresponding residue in TMEM16F was shown to be involved in phospholipid scrambling [48] (Figure 2g). The mutation K584Q in TMEM16A weakens the anion selectivity of TMEM16A and the corresponding mutation Q559K in TMEM16F its selectivity for cations [41•]. Strikingly, these residues are positioned within the hydrophilic groove, nearly midway between the cytosolic and luminal sides of the protein (Figure 2g). In three studies it has been attempted to create hybrid proteins of TMEM16 scramblases and TMEM16A that would gain the functional phenotype of the other protein. A first approach was performed in the Nagata lab, however the structural boundaries to fuse amino acid stretches from TMEM16A and TMEM16F were unknown at that time and the results were thus difficult to interpret [48]. Later, with structural information in hand, the Hartzell lab succeeded in converting TMEM16A into a scramblase by introducing sequence elements (the 'scrambling domain' SCRD) of TMEM16F [49•] (Figure 2h). The minimal stretch to convey scrambling activity to TMEM16A was only 15 amino acids long, contains part of α-helices 4 and 5 and is located within the hydrophilic groove of the subunit cavity. In a recent study this approach was used to identify the SCRD in the equivalent region of TMEM16E, a protein that, due to its intracellular localization, was difficult to investigate [50]. Together, these experiments emphasize the importance of this hydrophilic seam for lipid scrambling. It is nevertheless still necessary to experimentally confirm the passage of headgroups through the subunit cavity [30••].

Substrate specificity and lipid transfer rates of TMEM16 scramblases

Whereas scramblases are generally not specific, there must be restrictions with respect to size and chemical properties of the transported lipids. The concept of a slide-like facilitated diffusion of lipid headgroups on the polar surface of the subunit cavity, as discussed here, implies that the alkyl moieties of the lipids probably play a minor role in the process. To date, however, the influence of fatty acids and their degree of saturation has not been investigated in detail. Various phospholipids have been shown to be shuttled *in vitro* by nhTMEM16 and afTMEM16 without major differences [30••,42••] (Figure 3a and b). For some TMEM16 members clear lipid preferences were measured, e.g. TMEM16C prefers PC over Galactosylceramide (GalCer),

while PS is not transported at all [29••]. The spacious subunit cavity, with an average diameter of 6-12 Å, makes even the transfer of bulkier lipids plausible. Investigations of, at that time unassigned, erythrocyte scramblases has revealed that headgroups bearing trisaccharides could still be transferred but they are close to the upper size limit [31]. It has also been shown that glycosylceramides are substrates of certain TMEM16 scramblases [29••,42••]. Since ceramides do not contain any phosphate, this finding argues against the phosphate as a mandatory component of a lipid to be recognized as a substrate. It is important though, that ceramides have naturally a much higher flip-flop rate than phospholipids with a half-time of minutes, which suggests that the energy barrier for trans-bilayer movement is in this case lower [51,52]. In several studies, slow ion conduction was observed in TMEM16F [39,43], and it has been suggested that this may be a consequence of scrambling [30••,49•]. Whether the expected deformation of the bilayer near the entry sites of the conducting trench causes ion leak [30••,49•], or the efficient transfer of lipids even requires the parallel transport of counterions is currently not clear. Cation conduction observed for TMEM16F could thus compensate for the net charge of lipids on their way across the membrane. If the current is linked to lipid movement, it is conceivable that ion conduction in the absence of scrambling observed in TMEM16A/B evolved as a further specialization of this process. Indeed, it has recently been proposed that lipids might be part of the ion conduction pore of TMEM16A as a relic of an ancestral scramblase [53].

Lipid transfer rates

In contrast to slow ATP driven flippases that function by an alternate access mechanism, the transport of lipids by TMEM16 scramblases does not rely on conformational changes in the protein, and is consequently fast. Based on *in vitro* experiments the transfer rate has been estimated as 10^4 molecules/s [42••] and simulations suggested even orders of magnitude faster rates [54]. To achieve such a high turnover it might be a requirement to transfer several lipids at once in a concerted manner. The architecture of the subunit cavity appears suitable to allow for the passage of lipids that are lined up in a single file across the membrane [30••] (Figure 3c). A similar mechanism as has also been proposed from coarse-grained molecular dynamics simulations [54].

The calcium binding site: Potential mechanisms of activation

TMEM16 proteins are activated by sub-micromolar Ca^{2+} concentrations, a shared characteristic of channels and scramblases [30••,37-39,55]. The underlying regulatory ligand binding site has been identified in the nhTMEM16 structure [30••], but some of the interacting residues have previously been found in functional studies [47•,56]. In the structure of nhTMEM16 (crystallized in the presence of 3 mM Ca^{2+}), each subunit shows density for two Ca^{2+} ions that bind at a distance of 4.2 Å from one another. The ions directly interact with six conserved residues, five of which carry a negative charge. These residues are contributed by α-helices 6, 7 and 8 and are placed close to the border of the hydrophilic seam of the subunit cavity (Figure 3d, e and f). The location of this site within the transmembrane region likely explains the voltage dependence of Ca^{2+}-activation that was originally observed in the channel TMEM16A [30••,38,39,55]. Mutations of interacting residues impair activation of channels and scramblases [30••,41•,42••,47•,56,57] (Figure 3b). *In vitro* experiments of reconstituted nhTMEM16 and afTMEM16 in liposomes show a clear calcium-dependence of scrambling, but low activity was also measured without addition of Ca^{2+} in the presence of EDTA [30••,42••] (Figure 3b). This effect might be due to residual Ca^{2+} ions still bound to the protein, which is supported by a structure crystallized under apparent ligand-free conditions that still contained Ca^{2+} ions in the binding site [30••]. Alternatively, TMEM16 scramblases might also show residual activity in the absence of Ca^{2+}. This would be masked inside cells due to the constant activity of flippases and floppases and therefore not be disruptive for the asymmetry of the bilayer. Interestingly, the TMEM16F mutant D409G, which was key for identifying TMEM16F as a scramblase [28••], is already activated at resting Ca^{2+} concentrations [28••,43]. The basis for this effect is currently unknown since the location of the mutation is remote from the Ca^{2+} binding site. Still, it is noteworthy that this residue is located in proximity to a stretch of amino acids that in TMEM16A was identified to influence Ca^{2+} sensitivity [55,58,59]. The major question of how Ca^{2+} ions activate scrambling is currently unknown. We thus can only speculate about potential mechanisms (Figure 4): First, a structural rearrangement in the hydrophilic groove might take place to block the passage of lipids ("clogging"). Second, the N-termini might plug the cytosolic entry of the seam by a movement towards the membrane ("plug") and third, no large conformational change takes place, but instead the negative charges in the Ca^{2+} free form serve as an electrostatic gate for phospholipids. Interestingly the Ca^{2+} binding site is located such that the negatively charged phosphate of the lipid headgroup might experience repulsive forces upon entering the cavity. A neutralization of the charge upon Ca^{2+} binding would consequently facilitate lipid movement. However, scrambling of ceramides (that

do not contain a negative charge) still requires activation of the scramblase by Ca^{2+} [29••,42••]. Also, combinations of the abovementioned scenarios are conceivable.

Other scramblases

The field investigating proteins with scrambling function is still poorly charted and our current knowledge is limited. Recently, certain members of the family of Xk-related proteins (Xkr) have been identified as scramblases. Xkr8 and the *C. elegans* homologue CED-8 were shown to be the responsible factor for PS-externalization in apoptotic cell death, in conjunction with synchronous caspase-mediated inactivation of flippases [27•,60]. They are activated by cleavage of the termini by caspase-7 (in *C. elegans* CED-3) [27•]. It has been demonstrated that mutation within the cleavage site abrogated proteolysis and PS exposure [27•]. Within the family, Xkr4 and 9 also function as scramblases and like Xkr8 they locate to the plasma membrane [61]. Whether these proteins share similar structural features with TMEM16 scramblases is currently unknown. Xkr proteins have been predicted to contain 6-10 transmembrane spanning domains and, similar to TMEM16 proteins, some of these helices are relatively hydrophilic. A confirmation of their activity by functional reconstitution is still lacking. A number of scramblases in the ER still await molecular identification, such as the proteins responsible for the flip-flop of components for N-glycosylation [20,62,63] and GPI-anchoring [64], or the ER-lipid equilibrator required for membrane growth [20,63]. A candidate protein has been identified for the dolichol-flippase activity [65], but these assignments are still controversial [66,67]. Recently, rhodopsin was also associated with scrambling processes. However, compared to TMEM16 proteins, its structure and activity profile suggests a fundamentally different scrambling mechanism [68-70].

Conclusions

The structure of a TMEM16 protein has provided first insight into the architecture of scramblases and it has paved the way for detailed structure-based biophysical investigations of lipid scrambling. In line with previous predictions, the structure contains a hydrophilic cleft, the ‘subunit-cavity’, that is exposed to the membrane and that spans the entire width of the bilayer. Since this cleft is of appropriate size to accommodate their headgroups, lipids may diffuse along this path on their way across the membrane. Alternatively, also other mechanisms to lower the barrier for lipid flip-flop are conceivable (see for example [68]). The structure has revealed a regulatory calcium binding site that is located within the membrane, in proximity to

the subunit cavity, but the mechanism by which Ca^{2+} activates the protein is still ambiguous. From a biophysical perspective, the mode of calcium activation and the detailed analysis of lipid transport are major areas of future research on this protein family.

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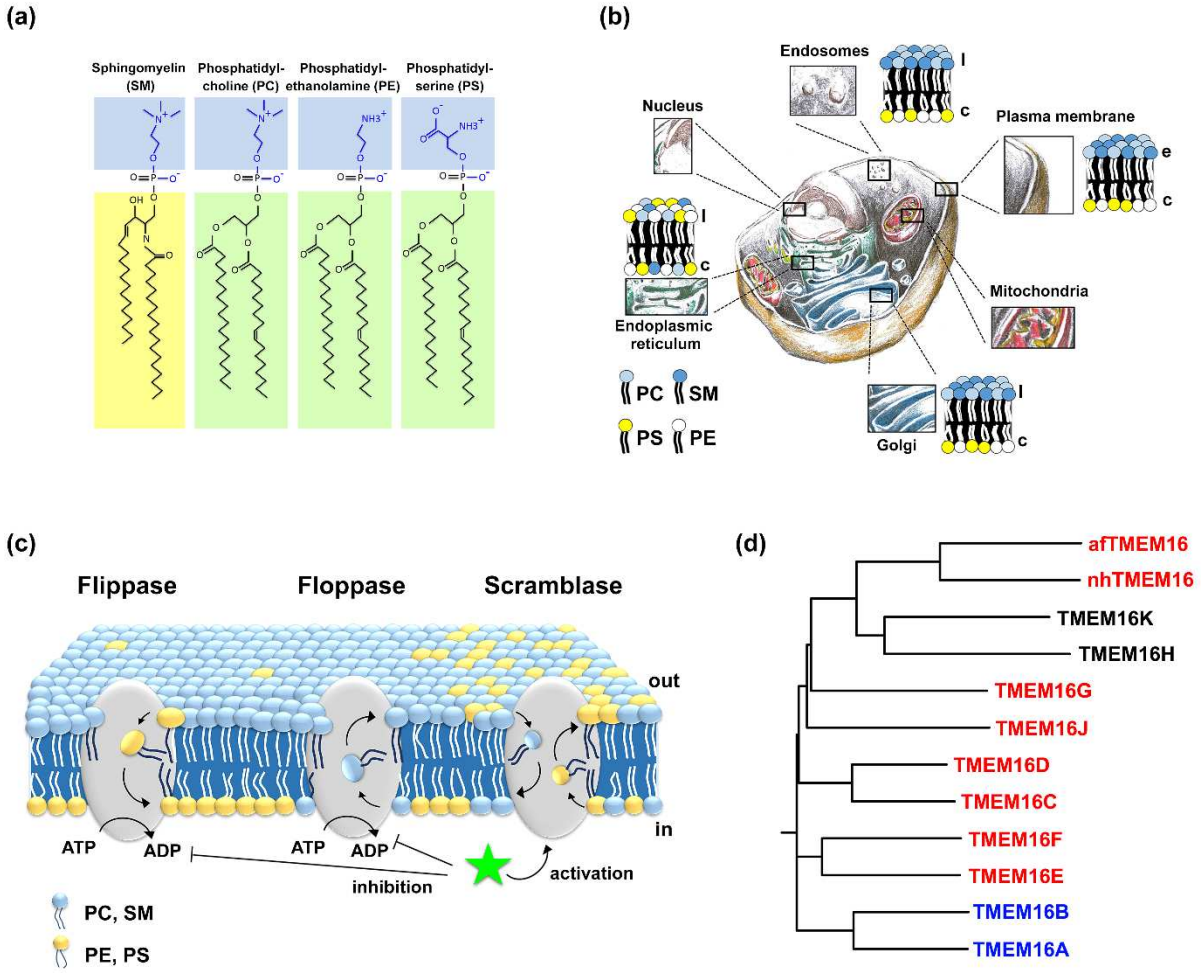

Figure 1

Lipids and lipid transport proteins. (a) Major phospholipids of an animal cell. The polar headgroups are highlighted in blue, the sphingosine backbone of SM in yellow and the glycerophospholipid backbone in green. (b) Schematic illustration of a eukaryotic cell and its organelles. The membrane composition and degree of membrane asymmetry is indicated for certain compartments. While the membrane composition is asymmetric at the plasma membrane, in the Golgi and late endosomes, the lipid distribution in the ER is thought to be random. l refers to lumen, c to cytoplasm, e to extracellular side. (c) Lipid transport. At the plasma membrane the lipid asymmetry is maintained by the collaborative action of ATP-dependent flippases and floppases. This asymmetry breaks down upon activation of scramblases, leading to the exposure of PS to the outer leaflet. The stimuli (green star) that cause the activation of scramblases typically have an inhibitory effect on flippases and floppases. (d) Phylogenetic tree of TMEM16 proteins, illustrating the relationship between human (TMEM16A-K) and fungal (afTMEM16 from *Aspergillus fumigatus* and nhTMEM16

from *Nectria haematococca*) family members. Family members with confirmed lipid scrambling activity are colored in red, ion channels in blue and members with yet uncharacterized function in black.

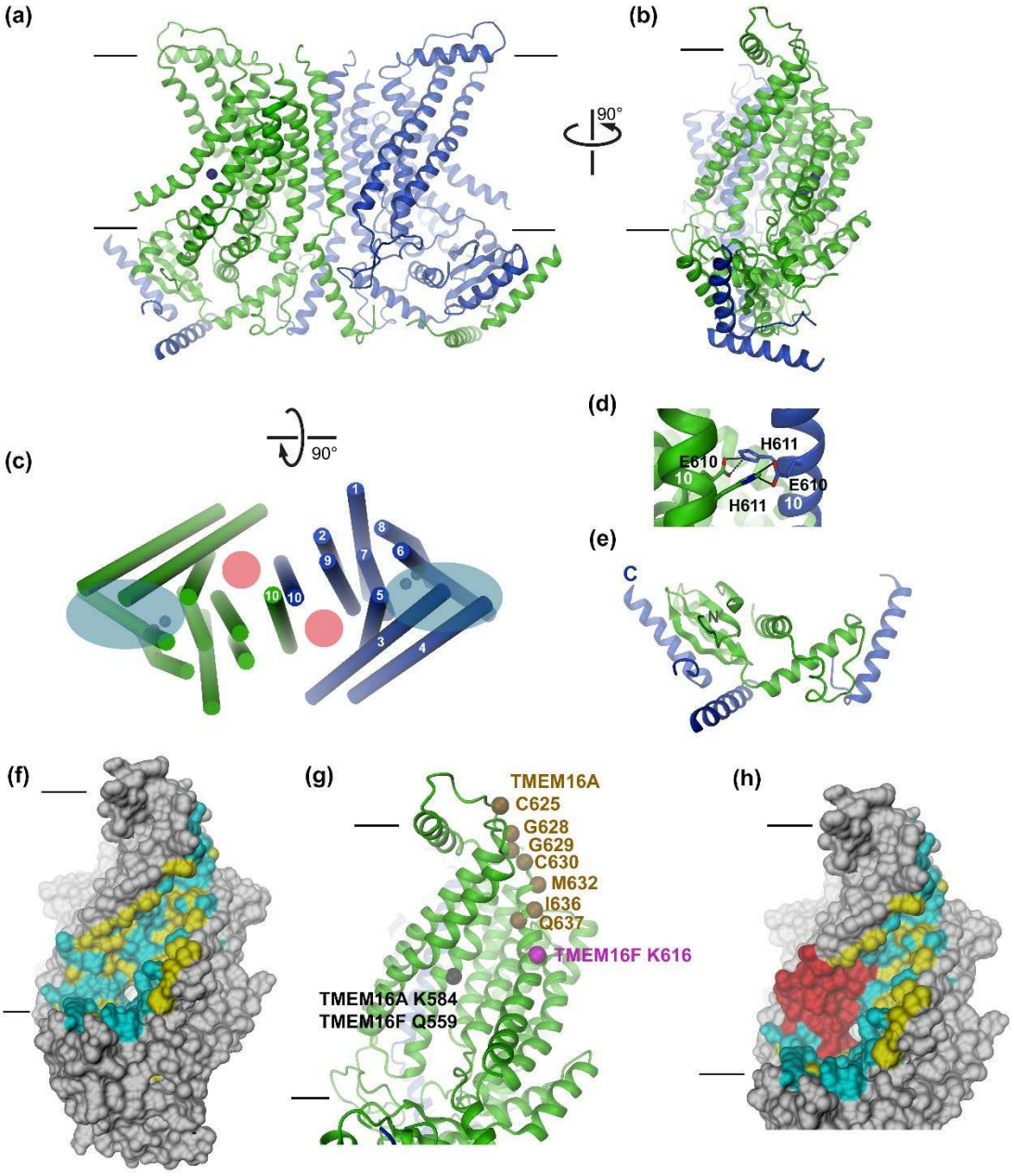

Figure 2

NhTMEM16 structure. (a) Ribbon representation of the nhTMEM16 dimer viewed from within the membrane. Bound Ca^{2+} ions are shown as dark blue spheres. (b) View on the subunit cavity. The orientation relative to (a) is indicated. (c) Organization of transmembrane helices in the

nhTMEM16 dimer. The view is from the extracellular side. Transmembrane helices are shown as cylinders. Selected helices are numbered. The locations of the dimer cavity (red) and subunit cavity (blue) are indicated. (d) Interaction between Glu610 and His611 connecting α-helices 10 at the dimer interface. Interacting residues that are conserved in TMEM16 scramblases but not in channels are shown as sticks. (e) Close-up of interacting N-and C-terminal domains of adjacent subunits. (f) Surface representation of nhTMEM16 highlighting the subunit cavity. Amino acids within the subunit cavity are colored according to their chemical properties (yellow: hydrophobic and aromatic; cyan: polar, acidic and basic). (g) Close-up of the subunit cavity with equivalent positions that alter the functional properties of TMEM16A and TMEM16F shown as spheres and labeled, (beige: positions that influence ion conduction in TMEM16A, black: position where mutations exert a pronounced effect on ion selectivity in TMEM16A and TMEM16F, magenta: Position equivalent to Lys616 in TMEM16F that was shown to play a role in lipid scrambling). (h) Close-up of a surface representation of the subunit cavity with residues that were replaced in a scrambling-competent TMEM16A-TMEM16F-TMEM16A chimera colored in red. Other residues are colored as in (f). In (f-h) the view is as in (b). In (a, b, f-h) the membrane boundary is indicated by black lines.

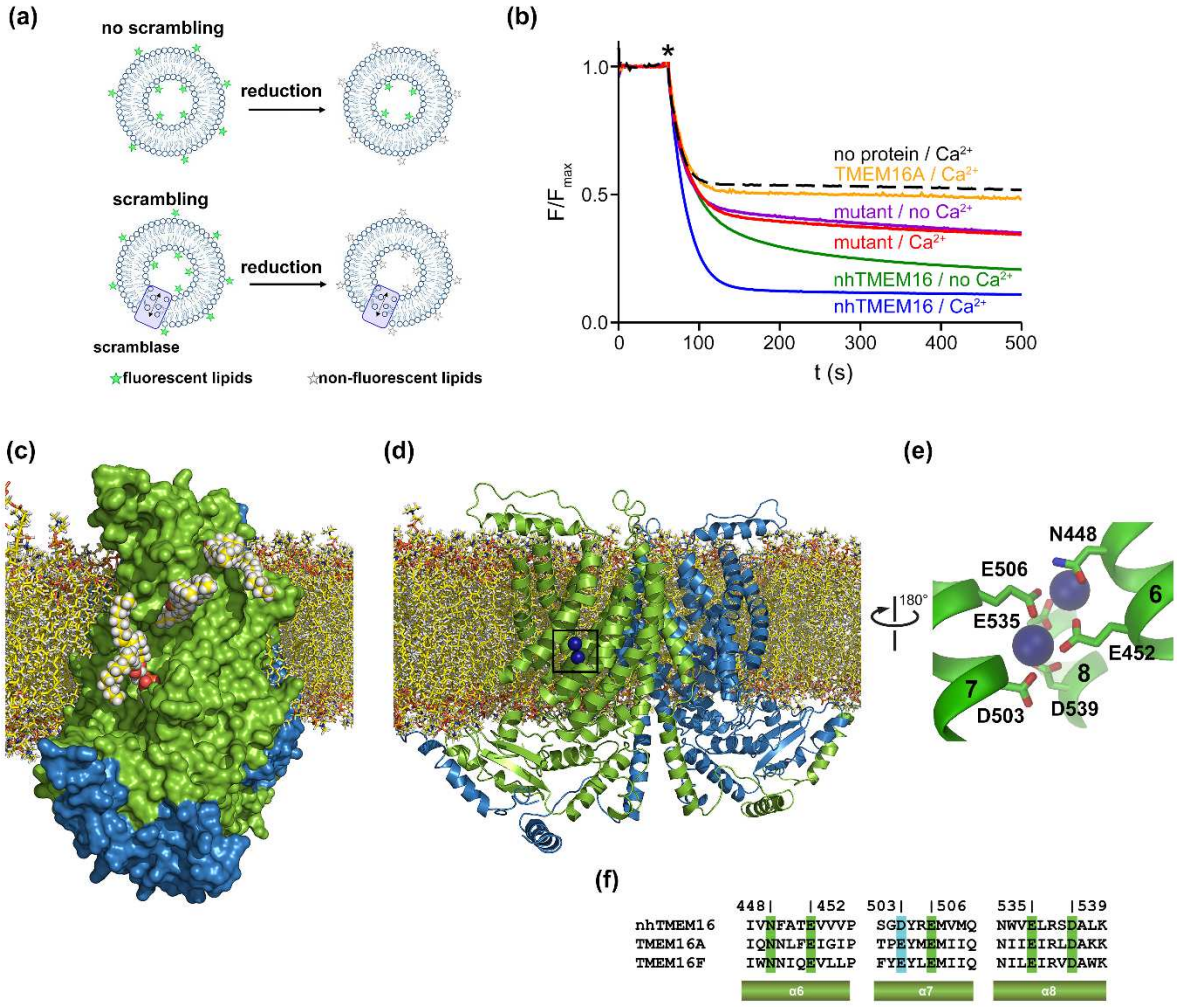

Figure 3

Ca^{2+} activation. (a) Schematic representation of the *in vitro* scrambling assay monitoring the dithionite-induced loss of fluorescence of NBD-conjugated lipids in the outer leaflet of artificial liposomes due to bleaching of the fluorophore. (b) Scrambling of NBD-PE. Representative traces of protein-free liposomes (no protein) or proteo-liposomes reconstituted with either TMEM16A, nhTMEM16 or nhTMEM16 carrying three mutations within the Ca^{2+}-binding site (E452Q/ E535Q/ D539N, mutant) in the presence or absence of Ca^{2+}. WT shows strong scrambling activity that is reduced in conditions where no Ca^{2+} is added. The Ca^{2+} binding site mutant shows strongly reduced activity that cannot be enhanced by the addition of Ca^{2+}. (c) View on the subunit cavity harboring phospholipid headgroups on their way across the membrane. The protein, shown as surface representation with subunits colored in green and blue respectively, is placed in a lipid bilayer model. Diffusing lipids within the subunit cavity

are shown as CPKs. Lipid positions are modeled. (d) Ca^{2+} binding site. Ribbon representation of nhTMEM16 placed into the model of a lipid bilayer with the position of the Ca^{2+} binding site of one subunit highlighted by a box. (e) Close-up of the Ca^{2+} binding site. The orientation with respect to (d) is indicated. The view is from within the subunit cavity. Conserved residues interacting with Ca^{2+} ions (blue spheres) are shown as sticks and labeled according to the nhTMEM16 sequence. (f) Sequence relationship of residues constituting the Ca^{2+} binding site in TMEM16 channels and scramblases. Interacting residues are highlighted (green identical, cyan homologous). Secondary structure elements are indicated.

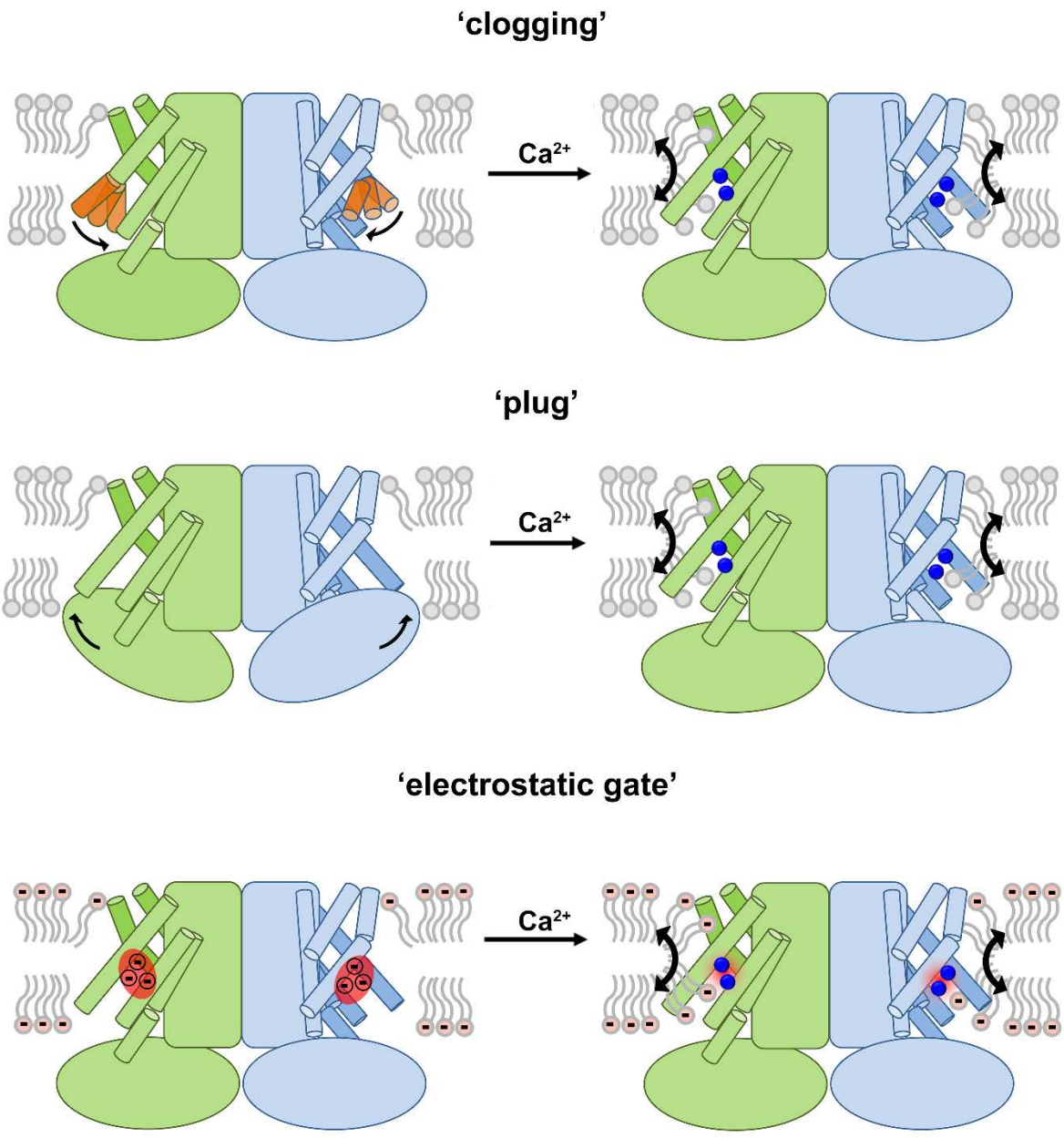

Figure 4

Potential mechanisms for Ca^{2+} activation. Inactive proteins are shown on the left, active proteins on the right. Ca^{2+} is indicated as blue spheres. Top, 'clogging', activation proceeds by a conformational change in the subunit cavity. Center, 'plug', the protein is activated by a movement of the cytoplasmic domains that block the path in the inactive conformation. Bottom, 'electrostatic gate', the Ca^{2+} neutralizes the excess negative net charge in the binding site (red) and this removes an electrostatic barrier for lipids traversing the cavity. This effect could be general for phospholipids (the negative charge of the phosphate at the lipid headgroup is indicated).

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- of special interest
- ●● of outstanding interest

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